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☐ *Additional inventors are being named on the ____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

COLLAGEN MIMETIC BIOMATERIALS

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 9 ☐ CD(s), Number

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☐ Application Data Sheet. See 37 CFR 1.76

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Respectfully submitted,

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Collagen Mimetic Biomaterials

Felicia A. Etzkorn, Xiaodong J. Wang, Matthew D. Shoulders

Field of Invention

This invention relates to the design and synthesis of collagen-like materials. More particularly the invention relates to materials that mimic the biological structure and behavior of collagen, yet are resistant to degradation.

Summary of the Related Art

Collagen is a highly abundant fibrous protein present throughout the human body, constituting approximately 25% of all protein in the body. Collagen is the scaffolding material found in skin, bones, tendons, cartilage, blood vessels and nearly all organs where it serves to form a matrix for holding and supporting cells. Collagen contains three polyproline type II helix chains each coiling in a left handed manner and coiling with each other to form a right-handed super helix. [1] The unique triple helical structure of collagen results from its primary structure, which can be represented as $(Xaa\text{-}Yaa\text{-}Zaa)_{300}$, where 10 percent of Xaa is proline, 10-12 percent of Yaa is 4(*R*)-hydroxyproline, and Zaa is typically Gly. [2,3] The presence of Gly at every third amino acid position is one of the most important structural elements of the collagen triple helix, as Gly is the only amino acid small enough to fit into the highly compacted super helix at that position. However, the high occurrence of hydroxyproline and proline in collagen and interchain hydrogen bonds between C=O and N-H groups contribute to stabilization of collagen's unique triple helical structure. [2,3] A typical molecule of collagen consists of around 300 units of Xaa-Yaa-Gly. This highly repeated sequence of collagen makes possible the polymerization of tripeptide monomers to prepare collagen analogues.

The existence of stable Xaa-Pro and Xaa-Hyp cis and trans amide conformational isomers leads to a significant challenge for folding collagen peptides. [4-8] In native collagens, globular C-terminal domains initiate triple helix formation, [9] but proline isomerization is still the slow step in collagen folding. [10] In an average 300 unit repeat of Xaa-Yaa-Gly, there are thus 600 amides that can exist in cis or trans. The number of possible conformational states of one strand is thus 2^{600}, and this does not include the necessity of triple helix formation. Folding of collagen occurs in a processive fashion. Once the triple helix is formed, the trans conformation is stable within the folded helix. Thus proline isomerization is rate limiting in collagen folding. [4-8]

Significant research has been performed regarding both the unique structural features of collagen and its potential biomedical applications. [11] Collagen is generally regarded as one of the most useful biomaterials due to its excellent biocompatibility and safety. Major uses of collagen as a biomaterial include applications of collagen in drug delivery systems and in tissue-engineering systems. However, the insufficient supply, poor mechanical strength, and ineffectiveness in the management of infected sites of collagen-based systems make improvements necessary. [12]

Several researchers have studied mimics of biological collagen, including polypeptides of the type $(Pro\text{-}Pro\text{-}Gly)_n$ and $(Pro\text{-}Flp\text{-}Gly)_n$ (where Flp represents 4(*R*)-fluoroproline), and all D-amino acid peptides. [13-15] However, none have prepared collagen mimics in which the amide bonds themselves have been altered.

The present invention is aimed towards preparation of self-assembling, biologically stable mimics of collagen via amide bond polymerization of appropriate monomers.

Disclosure of the Invention

The present invention relates to compounds with the general formula (**1A-3**, and Figure 1) which possess properties mimicking that of biological collagen and therefore have potential biomaterials applications.

(Gly-Ψ[*(E)*CH=C]-Xaa-Yaa)$_n$ **(1A)**

(Gly-Xaa-Ψ[*(E)*CH=C]-Yaa)$_n$ **(1B)**

(Gly-Xaa-Yaa-Ψ[*(E)*CH=CH])$_n$ **(1C)**

(Gly-Ψ[*(E)*CH=C]-Xaa-Ψ[*(E)*CH=C]-Yaa)$_n$ **(2A)**

(Gly-Xaa-Ψ[*(E)*CH=C]-Yaa-Ψ[*(E)*CH=CH])$_n$ **(2B)**

(Gly-Ψ[*(E)*CH=C]-Xaa-Yaa-Ψ[*(E)*CH=CH])$_n$ **(2C)**

(Gly-Ψ[*(E)*CH=C]-Xaa-Ψ[*(E)*CH=C]-Yaa-Ψ[*(E)*CH=CH])$_n$ **(3)**

The invention disclosure relates to the case **1A** where Xaa is Pro and Yaa is Hyp. However the scope of the invention includes the general case where any of the 20 natural amino acids are in the Xaa and Yaa positions or where Xaa and Yaa are represented by Hyp or Flp. These materials include block copolymers of alkene isostere monomers with tripeptide monomers, Figure 2. The enantiomeric case where all amino acids and their replacements have the unnatural D-amino acid, *R*- or *S*-stereochemistry at the α-position are also claimed (Figure 2). Such all D-amino acid analogues may have particular stability towards biological degradation with the enantiomeric right-handed triple helix supercoil producing similar macroscopic materials properties, yet interesting alternative biological properties. [15]

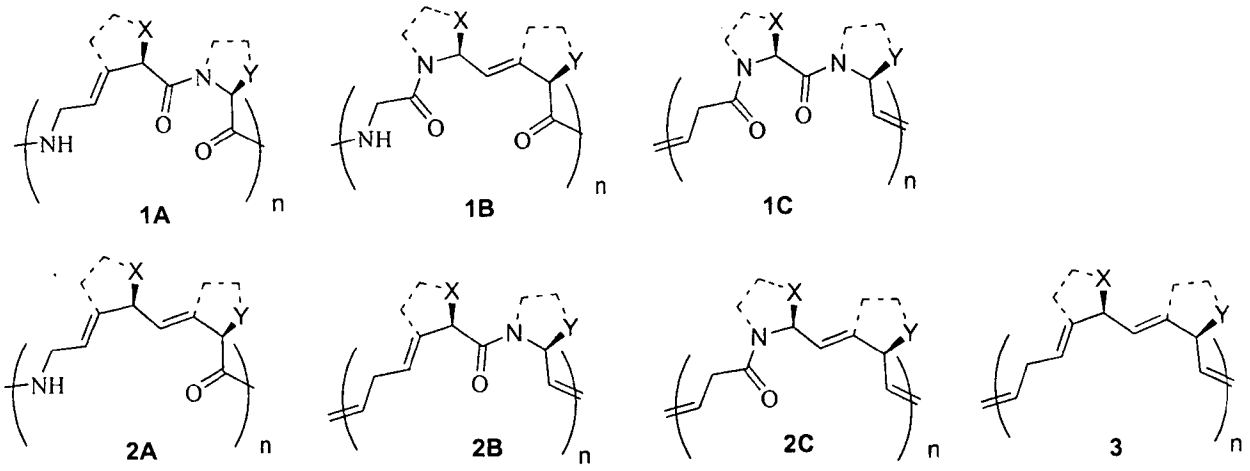
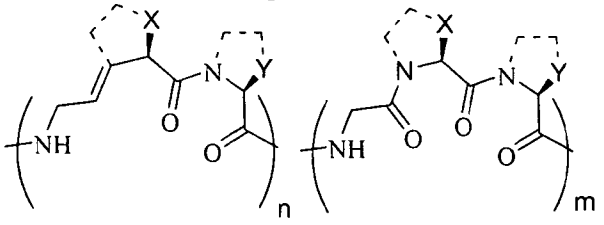


Figure 1. Structures of polymeric materials claimed.



example block copolymer with **1A**

Figure 2. Block copolymers of peptidomimetic with natural peptides.

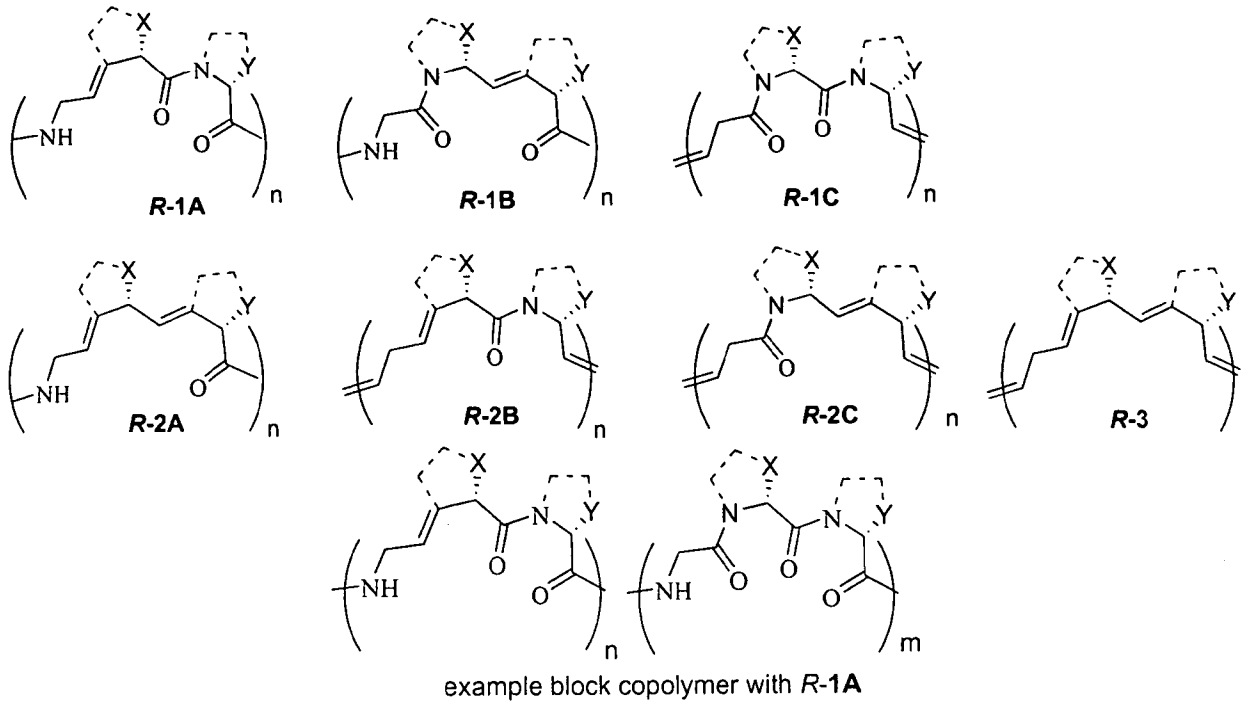


Figure 3. Enantiomeric collagen mimic materials claimed.

The structure of the specific monomer prepared is shown in Figure 4. This monomer may be polymerized via amide bond polymerization to form the desired collagen mimic.

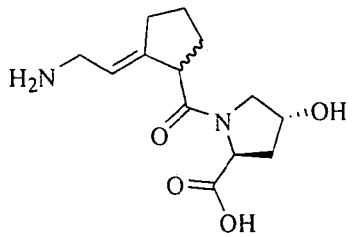


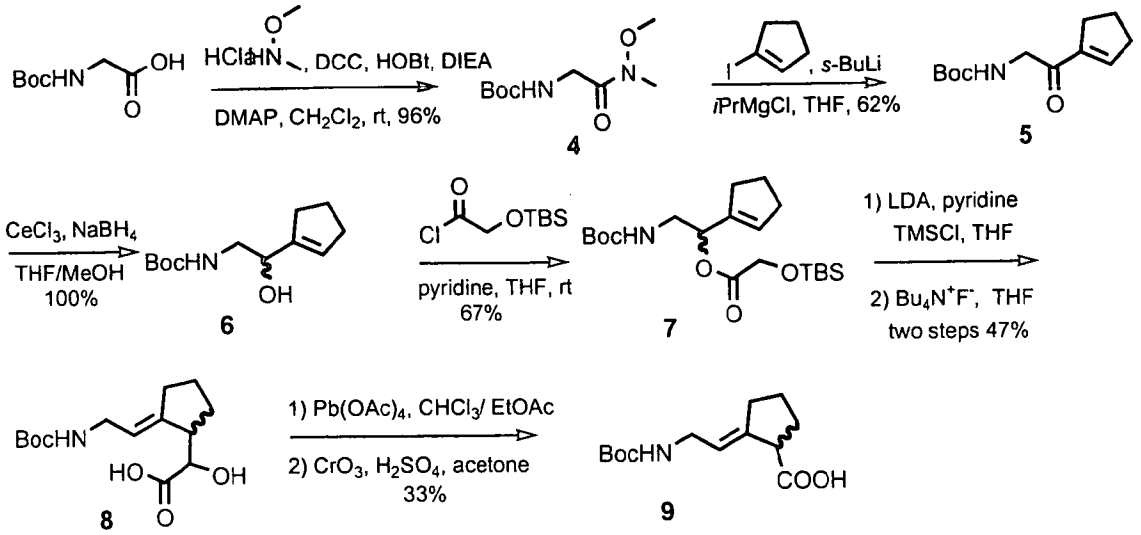
Figure 4. Structure of the monomer synthesized for the collagen mimic.

We will synthesize collagen-like peptides via polymerization of the monomer [Gly-Ψ[(*E*)CH=C]-Pro-Hyp]. Because all the Gly-Pro amide bonds in collagen exist in the trans conformation,[7,8] a route that affords the *E* monomer stereoselectively is desired. Our recent success in Ser-*trans*-Pro (*E*)-alkene isostere synthesis provides a route to this Gly-Ψ[(*E*)CH=C]-Pro-Hyp monomer. [16] Alkene amide bond surrogates provide not only conformational control but also resistance to peptidases. The alkene isostere material is also likely to inhibit collagenase (matrix metalloproteases), and may represent a method for preventing mucositis in cancer therapy,[17] or improving clinical markers of rheumatoid arthritis. [18] These synthetic collagen mimics will contribute not only to studies on the stability of collagen-like triple helical structures but will also provide useful structural biomaterials.

Synthesis of the [Gly-Pro-Ψ[*(E)*CH=C]Hyp]$_n$ Monomer

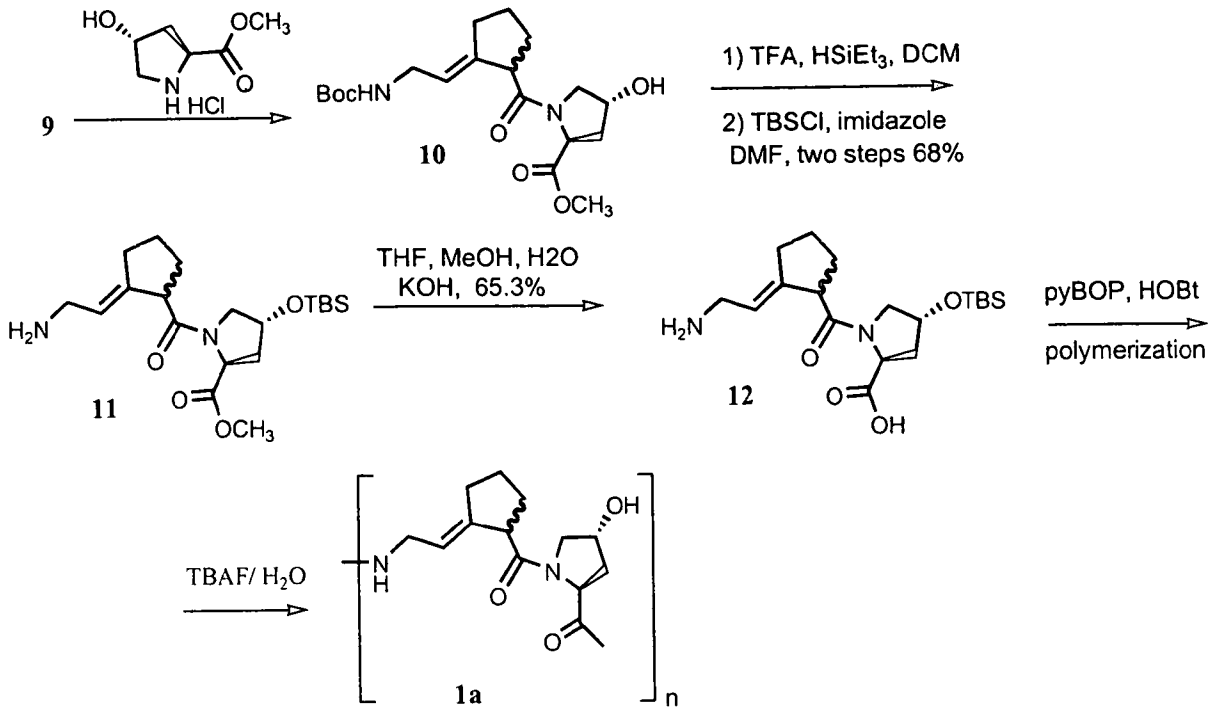
The synthetic scheme for preparation of the Gly-Ψ[(*E*)CH=C]Pro amide bond isostere is shown in Scheme 1 analogous to our previously described synthetic scheme. [16]

Scheme 1



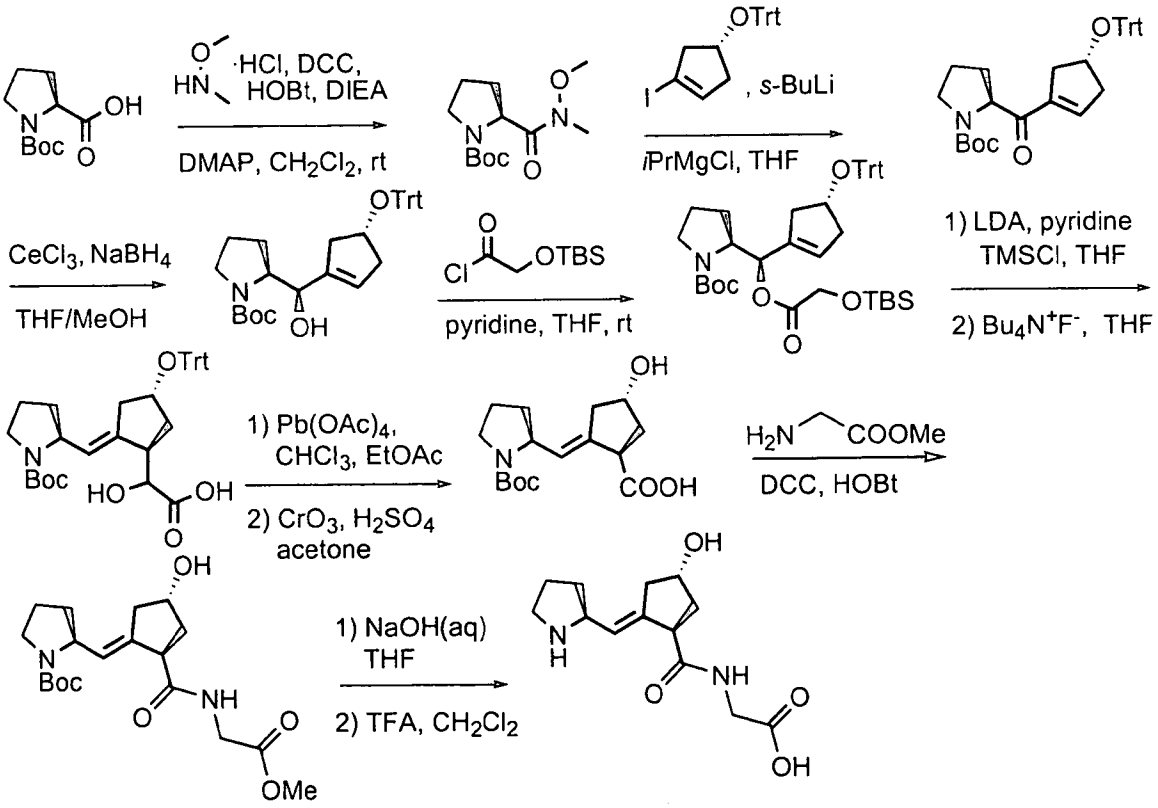
The general synthetic scheme displayed in Scheme 1 is applicable to all of the amino acids relevant to this invention as claimed above. In order to obtain a pure enantiomer of **9**, we will use a chiral hydrogenation catalyst instead of $CeCl_3$ in the reduction of α,β-unsaturated ketone **5**. We have used binaphthyl rhodium hydrogenation catalyst, but other catalysts are possible. After preparation of **9**, the monomer used for the amide bond polymerization may be prepared as displayed in Scheme 2.

Scheme 2



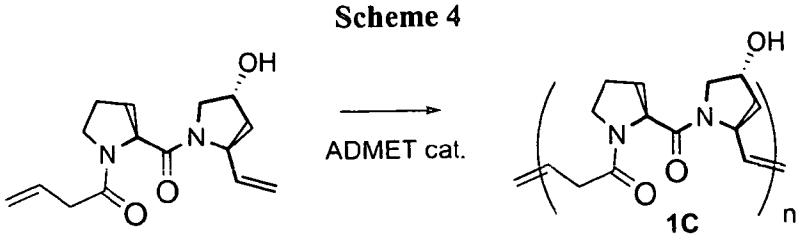
The synthesis of the monomer to make material **2B** is shown in Scheme 3. Polymerization can be performed by the method shown in Scheme 2.

Scheme 3



Synthesis of materials of type C, including **3**, will be polymerized by ADMET (acyclic diene metathesis) catalysis. [19] An example is shown in Scheme 4 for type **1C**.

Scheme 4



metathesis catalysts: Grubb's, Mol's, etc

Experimental

General. Unless otherwise indicated, all reactions were carried out under N_2 in flame-dried glassware. THF and CH_2Cl_2 were dried by passage through aluminum. Anhydrous (99.8%) peptide synthesis grade DMF, NMP and diisopropylethylamine (DIEA) were purchased from Fluka Chemical Co. for solid phase synthesis. Brine (NaCl), $NaHCO_3$, and NH_4Cl refer to saturated aqueous solutions unless otherwise noted. Flash chromatography was performed on 32-63 μm or 230-400 mesh, ASTM silica gel with reagent grade solvents. NMR spectra were obtained at ambient temperature in $CDCl_3$ unless otherwise noted. Proton and carbon-13 NMR spectra were obtained at 500 and 125 MHz, respectively. Coupling constants J are given in Hertz.

Boc-Gly Weinreb amide (4)[20] *N*-Boc-Gly-OH (10.5 g, 60.0 mmol), *N,O*-dimethylhydroxylamine hydrochloride (11.1 g, 120 mmol) and DIEA (31.2 g, 240 mmol) were dissolved in 1:1 CH_2Cl_2/DMF (500 mL) and cooled to 0 °C. 1-Hydroxy-1H-benzotriazole (HOBt, 11.0 g, 72.0 mmol), DCC (14.9 g, 72.0 mmol) and DMAP (ca. 100 mg) were added and the reaction was stirred for 24 h. The reaction was filtered to remove dicyclohexylurea and concentrated. The resulting slurry was diluted with 500 mL ethyl acetate and washed with NH_4Cl (2 × 100 mL), $NaHCO_3$ (2 × 100 mL) and brine (100 mL). The organic layer was dried on $MgSO_4$ and concentrated. Chromatography on silica with 20% EtOAc in hexane gave 12.6 g (96%) of **4** as a colorless plate-like crystal. m.p. 101-102 °C. ^{1}H NMR δ 5.25 (br, s, 1H), 4.07 (d, J=3.7, 2H), 3.70 (s, 3H), 3.19 (s, 3H), 1.44 (s, 9H).

Ketone (5). To a solution of 1-iodocyclopentene[16] (2.91 g, 15.0 mmol) in 80 mL THF at -40 °C was added *s*-BuLi (1.3 M in cyclohexane, 23 ml, 30 mmol). The reaction was stirred at -40 °C for 3 h to generate cyclopentenyl lithium. In another flask, Boc-glycine Weinreb amide **4** (2.18 g, 10.0 mmol) was dissolved in 20 mL of dry THF, degassed and inerted under N_2. The solution was cooled to -15 to -10°C and to the resulting slurry was charged 4.9 mL of 2.0 M *i*-PrMgCl/THF (9.8 mmol) dropwise at -15 to -5°C to afford a clear solution. After cooling to -78°C, the cyclopentenyl lithium solution was added via cannula to the deprotonated Weinreb amide solution. The mixture was stirred for 1 h at –78 °C, quenched with NH_4Cl (10 mL), diluted with EtOAc (100 mL), washed with NH_4Cl (2 × 20 mL), $NaHCO_3$ (20 ml), brine (20 mL), dried over $MgSO_4$ and concentrated. Chromatography on silica with 10% EtOAc in hexane gave 1.40 g (62%) of ketone **5** as a yellowish solid. ^{1}H NMR δ 6.81 (s, 1H), 5.36 (br, s, 1H), 4.29 (d, J=4.6, 2H), 2.56 (t, J=7.7, 4H), 1.92 (m, 2H), 1.43 (s, 9H). ^{13}C NMR δ 192.9, 155.8,

144.6, 143.1, 79.7, 47.5, 34.2, 30.6, 28.4, 22.5. Anal. Calcd. for: $C_{12}H_{19}NO_3$: C, 63.98; H, 8.50; N, 6.22. Found: C, 63.71; H, 8.51; N, 6.15.

Alcohol (6). Ketone **5** (1.35 g, 6.00 mmol) was dissolved in 2.5:1 THF/MeOH (70 ml) and cooled to 0 °C. $CeCl_3$ (2.69 g, 7.20 mmol) was added, followed by $NaBH_4$ (0.46 g, 12 mmol). After stirring 1 h at 0 °C, the reaction was quenched with NH_4Cl (15 mL), diluted with EtOAc (100 mL), washed with NH_4Cl (2 × 20 mL), brine (20 mL), dried on $MgSO_4$ and concentrated. Chromatography on silica with 20% EtOAc in hexane yielded 1.36 g (100%) of product as a white solid. ^1H NMR δ 5.66 (m, 1H), 4.89 (br, s, 1H), 4.31 (d, *J*=5.5, 1H), 3.38 (m, 1H), 3.13 (m, 1H), 2.31 (m, 4H), 1.88 (m, 2H), 1.43 (s, 9H) ^{13}C NMR δ 156.7, 144.6, 126.4, 79.6, 70.9, 45.3, 32.3, 31.9, 28.4, 23.4. Anal. Calcd for: $C_{12}H_{21}NO_3$: C, 63.41; H, 9.31; N, 6.16. Found: C, 63.63; H, 9.47; N, 6.09.

Ester (7). To a solution of alcohol **6** (12 mg, 0.053 mmol) and pyridine (13.3 μL, 0.165 mmol) in THF (0.1 mL) was added a solution of *t*-butyldimethylsilyloxyacetyl chloride[21] (12 mg, 0.055 mmol) in THF (0.1 mL) dropwise at 0 °C. The reaction was stirred for 0.5 h at rt then diluted with 5 mL Et_2O, washed with 0.5 N HCl (2 × 0.4 mL), $NaHCO_3$ (1 mL), brine (1 mL), dried on $MgSO_4$ and concentrated. Chromatography with 5% EtOAc in hexanes on silica gave 14.5 g (67%) of ester **7** as colorless oil. ^1H NMR δ 5.67 (s, 1H), 5.48 (br, s, 1H), 4.64 (br, s, 1H), 4.24 (s, 2H), 3.43 (m, 1H), 3.33 (m, 1H), 1.87 (m, 2H), 1.45 (s, 9H), 0.90 (s, 9H), 0.08 (s, 6H). ^{13}C NMR δ 171.2, 155.8, 139.9, 128.8, 79.6, 72.8, 61.8, 42.8, 32.4, 32.0, 28.4, 25.9, 25.6, 23.1, -5.4.

α-Hydroxy acid (8). To a solution of diisopropylamine (0.21 mL, 1.5 mmol) in THF (2.0 mL) was added *n*-butyl lithium (2.5 M in hexane, 0.54 mL, 1.3 mmol) at 0 °C. The mixture was stirred for 15 min to generate LDA. Then a mixture of chlorotrimethyl silane (0.46 mL, 3.7 mmol) and pyridine (0.32 mL, 4.0 mmol) in THF (0.8 mL) was added dropwise to the LDA solution at –100 °C. After 5 min, a solution of ester **5** (136 mg, 0.333 mmol) in THF (1 mL) was added dropwise and the reaction was stirred at –100 °C for 25 min then warmed slowly to rt over 1.5 h and heated to 45 °C for 1 h. The reaction was quenched with 1 N HCl (5.0 mL) and the aqueous layer was extracted with Et_2O (2 × 7 mL). The organic layer was dried on $MgSO_4$ and concentrated to give 106 mg (crude yield 78%) yellowish glassy oil. Without further purification, the product was dissolved in 0.8 mL THF. Tetrabutylammonium fluoride (261 mg, 1.00 mmol) in THF (0.5 mL) was added at 0 °C, stirred at 0 °C for 5 min then at rt. for 1 h. The reaction was quenched with 0.5 N HCl (2 mL), extracted with EtOAc (5 mL), dried on $MgSO_4$ and concentrated. Chromatography with 5% methanol in $CHCl_3$ on silica gave 46.2 mg (52%) of α-hydroxy acid **8** as yellowish oil. ^1H NMR (DMSO-d_6) δ 6.81, (br, s, 1H)), 5.31 (br, s, 1H), 3.84 (d, *J*=5.8, 1H) 3.48 (m, 2H), 3.16 (t, *J*=8.5, 1H) 2.64 (m, 1H), 2.27 (m, 1H), 2.12 (m, 1H), 1.70 (m, 2H), 1.58-1.42 (m, 2H), 1.37 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 175.4, 156.0, 144.4, 120.2, 79.7, 78.0, 73.7, 58.1, 47.4, 29.8, 28.9, 24.5, 23.6, 24.5, 23.6, 19.8, 14.1.

Acid (9). Lead tetraacetate (78 mg, 0.17 mmol) in $CHCl_3$ (0.4 mL) was added dropwise to a solution of acid **8** (45.6 mg, 0.16 mmol) in EtOAc (2.2 mL) at 0 °C. The reaction was stirred for 10 min, then quenched with ethylene glycol (0.6 mL), diluted with EtOAc (20 mL), washed with H_2O (4 × 2 mL) and brine (2 mL), dried on Na_2SO_4, and concentrated to give 38 mg (100% crude yield) of aldehyde as yellow oil. The product was dissolved in acetone (4.8 mL) and cooled to 0 °C. Jones reagent (2.7 M H_2SO_4, 2.7 M CrO_3; 0.12 mL, 0.32 mmol) was added dropwise. The reaction was stirred at 0 °C for 0.5 h, quenched with isopropyl alcohol (0.5 mL), and stirred for 10 min. The precipitate was filtered out, and the solvent was evaporated. The residue was extracted with EtOAc (3 × 5 mL), washed H_2O (1.5 mL) and brine (1.5 mL), dried

on Na_2SO_4, and concentrated. Chromatography on silica with 40% EtOAc and 0.1 acetic acid in hexane gave 12.5 mg (31%) of acid **9** as a white solid. ^1H NMR (DMSO-D_6) δ 12.16 (br, s, 1H), 6.93 (t, *J*=5.4, 1H), 5.37 (s, 1H), 3.50 (m, 2H), 3.16 (t, *J*=7.3, 1H), 2.29 (m, 1H), 2.22 (m, 1H), 1.80 (m, 3H), 1.55 (m, 1H), 1.37 (s, 9H). ^{13}C NMR (DMSO-D_6) δ 175.3, 156.1, 142.9, 120.8, 78.1, 49.5, 30.1, 29.2, 28.8, 25.0. Anal. Calcd for: $C_{13}H_{21}NO_4$: C, 61.16; H, 8.29; N, 5.49. Found: C, 61.11; H, 8.25; N, 5.48.

Amide (10): 1-Hydroxybenzotriazole (HOBt, 191.6 mg, 1.25 mmol), *N*-[(1H-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminiun hexafluorophosphate *N*-oxide (HBTU, 473.8 mg, 1.25 mmol), DIEA (3225/5 mg. 2/5 mmol) and acid **7** (119.6 mg, 0.5 mmol) were dissolved in DMF (25 mL), 4-Hydroxyproline methyl ester hydrochloride salt (224.5, 1.25 mmol) was added. The reaction mixture was stirred at rt for 1 h, then diluted with EtOAc (75 mL), washed with H_2O (3 × 25 mL), $NaHCO_3$ (25 mL), brine (25 mL), dried on $MgSO_4$ and concentrated. Chromatography with 50% EtOAc in hexanes yielded 110 mg of syrup.

Amine (11): Amide **10** (110 mg, 0.302 mmol) and triethylsilane (87.79mg, 0.755 mmol) were dissolved in 25% TFA in DCM and stirred for 0.5 h at rt. Solvent was removed by evaporation. Remaining TFA and triethyl silane was removed by vacuum. Without further purification, the residue was dissolved in 2 mL DCM, tert-butyldimethylsilyl chloride (91 mg, 0.604 mmol) and imidazole (82 mg, 1.208 mmol) was added. The reaction mixture was stirred at rt. For 4 h then dulited with EtOAc, washed with NaHCO3 (2 × 7 mL), H2O (7 mL), dried on $MgSO_4$ and concentrated. Chromatography on silica gel with 15% MeOH in chloroform gave 81 mg (67.7%) colorless oil.

Acid (12): To a solution of amine **11** (80 mg, 0.2 mmol) in THF (1.2 mL) was slowly added a solution of potassium hydroxide in 1:2 MeOH: H2O (0.6 mL) at -10 °C. After stirring for 1 h at 0 °C, the reaction was diluted with 5 mL THF, acidified with 1 N HCl (0.21 mL), dried over $MgSO_4$ and concentrated. Chromatography on silica gel with 15% MeOH in $CHCl_3$ yielded 50 mg (yield 65.3%) of colorless oil.

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